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Repression of the Transcription Factor Bach2 Contributes to Predisposition of IgG1 Memory B Cells toward Plasma Cell Differentiation

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SUMMARY

Memory B cells are essential for generating rapid and robust secondary antibody responses. It has been thought that the unique cytoplasmic domain of IgG causes the prompt activation of antigen-experienced IgG memory B cells. To assess this model, we have generated a mouse containing IgG1 B cells that have never encountered antigen. We found that, upon challenge, antigen-experienced IgG1 memory B cells rapidly differentiated into plasma cells, whereas nonexperienced IgG1 B cells did not, suggesting the importance of the stimulation history. In addition, our results suggest that repression of the Bach2 transcription factor, which results from antigen experience, contributes to predisposition of IgG1 memory B cells to differentiate into plasma cells.

INTRODUCTION

Humoral memory is characterized by the rapid production of high titers of high-affinity antigen-specific antibodies, mostly of the IgG isotypes in the systemic immune system. During their generation, memory B cells could acquire new traits that make them intrinsically different from their naive predecessors, which in turn, at least partly, contribute to the rapid activation of memory B cells (Good-Jacobson and Shlomchik, 2010). Indeed, memory B cells have been demonstrated to have a distinct gene expression profile that differs from naive B cells (Bhattacharya et al., 2007; Tomayko et al., 2008). However, which molecule(s) among these changes is critical to make the memory B cells better to respond to secondary antigen exposure remains unclear.

One obvious inherent difference between naive and memory B cells is in the structure of the B cell antigen receptor (BCR) (i.e., membrane IgM and IgD on naive B cells versus membrane IgG on memory cells), and this has long been suspected to be the molecular basis for the robust secondary antibody responses of memory B cells expressing IgG (BCR-intrinsic mechanism) (Engels et al., 2009; Liu et al., 2010; Martin and Goodnow, 2002). Membrane IgM and IgD both have short, three-amino-acid heavy-chain cytoplasmic tails that seem incapable of playing any direct role in BCR signaling. By contrast, all membrane IgG subclasses have unique cytoplasmic domain structures of 28 residues and these are highly conserved among species. Thus, in addition to BCR signaling executed by the Igα and Igβ heterodimer common to all BCRs, the IgG-type BCRs could possess additional signaling functions mediated by the cytoplasmic domain of IgG (Reth, 1992).

Indeed, in vitro murine studies show that cross-linking of IgM-type and IgG-type BCRs induced different signaling activity (Engels et al., 2009; Liu et al., 2010; Wakabayashi et al., 2002). In vivo studies have also demonstrated the potential of the unique function of the IgG1 tail. Mice with a targeted disruption that truncates the cytoplasmic sequence of IgG1 generate poor antigen-specific IgG1 secondary antibody responses (Kaisho et al., 1997). However, in these mice, the B cells expressing a truncated IgG1 have lower cell surface BCR expression than do wild-type IgG1-bearing B cells, raising the possibility that the reduced IgG1 antibody responses result from decreased stability of membrane IgG1 expression, rather than from a specific signaling defect. Another study uses transgenic mice harboring a chimeric membrane heavy chain consisting of the extracellular domain of IgM (anti-hen egg lysozyme) and the cytoplasmic domain of IgG1 (IgM-IgG1 BCR) (Martin and Goodnow, 2002). The B cells expressing an IgM-IgG1 BCR give rise to ten times more extrafollicular plasma cells than do B cells expressing an IgM BCR, demonstrating the unique capability of the IgG1 tail. However, these studies focus on the primary perifollicular and extrafollicular response. Given that authentic memory B cells are antigen experienced and many of them are derived from germinal center (GC) reactions (Good-Jacobson and Shlomchik, 2010), these experiments do not address the effect of the IgG1 tail on the activity of post-GC authentic memory B cells. Therefore the above studies, although suggestive, do not allow us to draw firm conclusions regarding the issue of

whether the IgG cytoplasmic domain on memory B cells is sufficient to account for their unique traits.

Indeed, because the initial priming with antigen causes numerous interconnected changes in the antigen-experienced IgG1 memory B cells, other BCR-extrinsic changes have been proposed to be important, mainly based on the in vitro analysis of human memory B cells. For instance, expression of KLF4, KLF9, and promyelocytic leukemia zinc finger (PLZF) transcription factors, which are important in maintaining cellular quiescence, is downregulated in both human IgM- and IgG-type memory B cells, and enforced expression of these genes in memory B cells delays their entry into cell cycle (Good and Tangye, 2007).

Here, as a first step to identify a causal factor for the unique traits of IgG1 memory B cells, we examined the contribution of BCR-intrinsic and -extrinsic changes in rapid recall responses. Our data suggest that antigen experience induces repression of the Bach2 transcription factor, which in turn contributes to the heightened differentiation activity of IgG1 memory B cells.

RESULTS

Importance of IgG1 Memory B Cells in Secondary Antibody Responses

To address the mechanisms underlying the rapid and robust recall responses, we used as a model system the well-characterized antibody (Ab) response to the hapten nitrophenol (4-hydroxy-3-nitrophenylacetyl [NP]) (Bothwell et al., 1981).

Because recent studies have raised the possibility that IgM-type memory B cells are able to generate a secondary antigen-specific IgG1 Ab response (Dogan et al., 2009; Pape et al., 2011), we first sought to determine the relative contribution of IgM- and IgG1-type memory B cells to secondary anti-NP IgG1 Ab responses. To specifically deplete IgG1-expressing cells, we used Cγ1-*cre* × iDTR mice, in which the *cre* recombinase gene is "gene targeted" to the Cγ1 locus so that Cre is expressed in cells transcribing the γ1 constant region, resulting in expression of the human diphtheria toxin receptor (DTR) and sensitivity to diphtheria toxin (Buch et al., 2005; Casola et al., 2006). Mice were immunized with NP-chicken-γ-globulin (CGG) in alum to generate NP-specific memory B cells and then rested for 60 days, at which time most of the GC B cells were reduced. The mice were then treated with diphtheria toxin for 5 days to delete preexisting IgG1-expressing B cells before secondary NP-CGG immunization. After the toxin treatment, compared with Cγ1-*cre* control mice, ~10% of $IgG1^+$ B cells were present in the Cγ1-*cre* × iDTR mice (Figure 1A, left). CD38 is a good marker for distinguishing between memory and GC B cells in mice; the former are $CD38^+IgG1^+$ and the latter are $CD38^-IgG1^+$ (Ridderstad and Tarlinton, 1998; Takahashi et al., 2001). Indeed, NP-specific $CD38^+IgG1^+$ memory B cells were efficiently deleted (Figure 1A, right). This significant reduction resulted in functional consequences, as shown by the fact that the diphtheria toxin-treated Cγ1-*cre* × iDTR mice were unable to generate a secondary anti-NP high-affinity IgG1 Ab response (Figure 1C). To confirm that this defect was directly due to the absence of IgG1 memory B cells, we transferred wild-type (WT) IgG1 memory B cells into the toxin-treated mice and were able to recover the secondary response. NP^+IgM^+ B cells were intact in toxin-treated Cγ1-*cre* × iDTR mice (Figure 1B), as was the secondary IgM anti-NP response (Figure S1 available online). Thus, we conclude that IgG1 memory B cells are the major source of the secondary IgG1 Ab response to NP.

IgG1 Memory B Cells Have an Enhanced Capacity to Differentiate into Plasma Cells

To define the unique features of IgG1 memory B cells that could explain the rapid secondary Ab response, we first compared the properties of IgG1 memory and IgM naive B cells. To do this, we chose an experimental system with a defined affinity for NP, because BCR affinity is known to be a critical determinant in B cell fate decision (Chan et al., 2010), such as in the choice between extrafollicular plasmablast responses versus GC pathways (Paus et al., 2006). Therefore, we made use of Ig V_H186.2-DFL16.1-J_H2 gene-targeted mice ($B1\text{-}8^{hi}$) in which the B cells express Ig heavy chains that, when combined with an Igλ light chain, produce Abs with a defined affinity for the hapten NP (Shih et al., 2002). These high-affinity $B1\text{-}8^{hi}$ B cells have a 10-fold higher affinity (Ka; $5 \times 10^6\ M^{-1}$) for NP than do germline B1-8 B cells (Ka; $5 \times 10^5\ M^{-1}$) because of the introduction of a Trp to Leu mutation at codon 33 of V_H186.2 (Allen et al., 1988).

To prepare $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells, $B1\text{-}8^{hi}$ IgM B cells were transferred into naive C57BL/6 mice, which were then immunized with NP-CGG in alum (Figure 2A). The $B1\text{-}8^{hi}$ memory B cells derived from $B1\text{-}8^{hi}$ IgM naive B cells, which could be easily distinguished from recipient B cells on the basis of CD45.1 or CD45.2 expression, were then purified. As reported with endogenous NP^+ IgG1 memory B cells (Anderson et al., 2007; Tomayko et al., 2010), these $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells expressed higher CD80, CD273, and major histocompatibility complex class II molecules (MHC-II) than $NP^+B1\text{-}8^{hi}$ IgM naive B cells (Figure 2B).

We then transferred equal numbers of IgM naive or IgG1 memory B cells into CGG in alum-primed C57BL/6 mice. Because $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells exhibited the mature follicular phenotype ($CD23^{hi}CD21^{int}$), we used the mature follicular fraction of the $B1\text{-}8^{hi}$ IgM naive B cells for this assay (Figure 2A). Recipients were immunized 24 hr later with NP-CGG in PBS, and spleen cells were examined by multiparameter flow cytometry on day 4 to follow the fates of the $NP^+B1\text{-}8^{hi}$ IgG1 memory and $NP^+B1\text{-}8^{hi}$ IgM naive B cells. There were several notable differences. The $NP^+B1\text{-}8^{hi}$ IgM naive B cells gave rise to more progeny cells than did the $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells (Figure 2C) and, based on Fas and GL7 expression, ~60% of the naive B cells had become GC cells, whereas few germinal center cells were generated by $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells (Figure 2D). By contrast, the memory B cells generated a high frequency of plasma cells (~55%) (Figure 2E), which is consistent with previous reports (Benson et al., 2009; Dogan et al., 2009; Pape et al., 2011).

In order to obtain sufficient NP^+ IgG1 memory B cells, we had to adoptively transfer large numbers of antigen-specific B cells, and therefore it could be argued that elevating the frequency of these cells introduced unknown variables that are not representative of the normal physiologic state. To validate the use of the adoptive-transfer system, we purified endogenous anti-NP-specific IgG1 memory B cells from normal C57BL/6 mice. We analyzed somatic hypermutation and affinity maturation by single-cell PCR and sequencing V_H186.2. About 20% of the

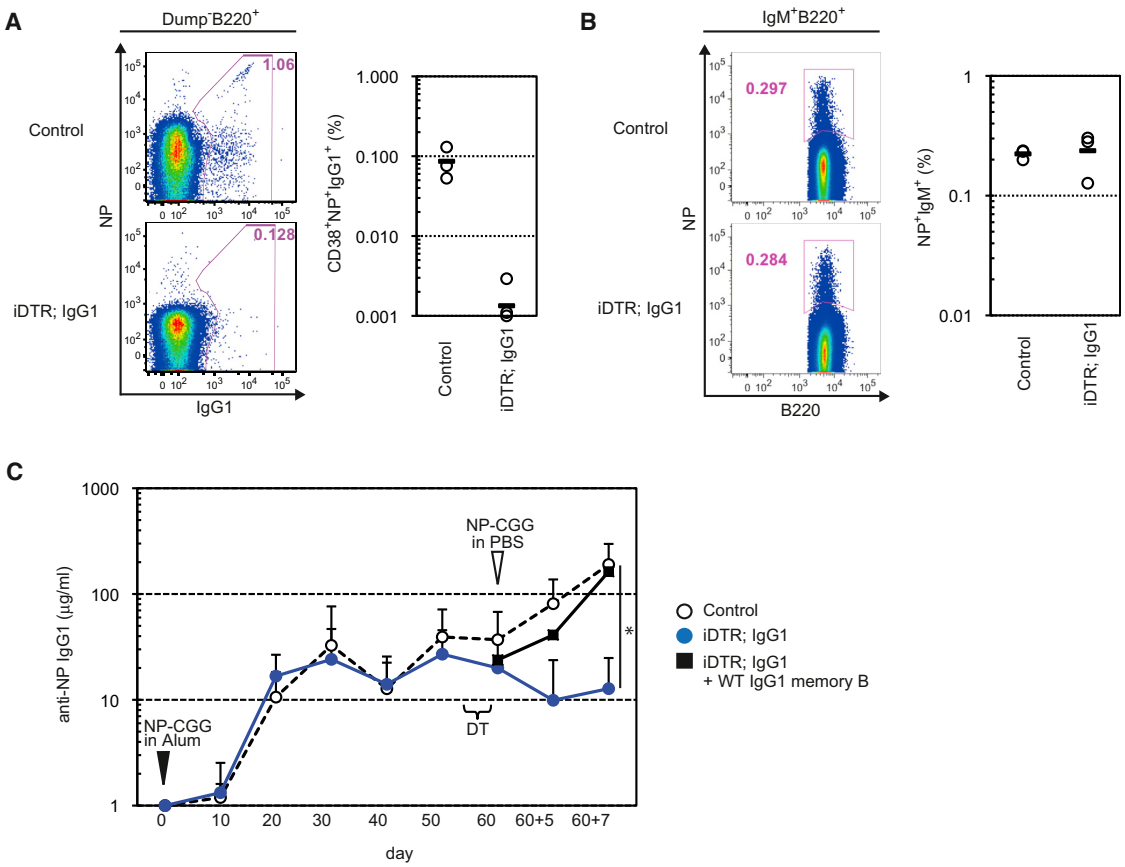

Figure 1. Secondary High-Affinity IgG1 Responses Are Derived Mainly from IgG1 Memory B Cells

Cγ1-*cre* gene-targeted mice (control) or Cγ1-*cre* × iDTR (iDTR; IgG1) mice were immunized with NP-CGG in alum. After 56 days, diphtheria toxin (DT) was injected intraperitoneally (i.p.) for 3 consecutive days. Two days after the last injection, splenocytes were prepared from each mouse strain.

(A and B) The percentage of NP-specific IgG1 memory B cells ($NP^+IgG1^+CD38^+$) in the spleen (A) and NP-specific IgM B cells (NP^+IgM^+) (B) were measured by flow cytometry. Numbers in each profile indicate the percentage of the gated population. Circles in the right hand plots indicate data from individual mice, and the bar indicates the mean.

(C) Serum of each mouse was collected at the indicated time points. Soluble NP-CGG was injected i.p. at day 60 after the primary immunization. Sera were collected at 5 days and 7 days after the rechallenge. Anti-NP high-affinity IgG1 antibodies in sera were measured by ELISA. NP-specific $IgG1^+CD38^+$ wild-type memory B cells were collected from NP-CGG in alum-immunized C57BL/6 mice and transferred to DT-treated iDTR; IgG1 mice at day 59. On the next day, recipient mice were boosted with soluble NP-CGG and anti-NP IgG1 titer was determined. Each group consists of more than three mice and representative data of two independent experiments are shown. Data represent the mean ± SD. *$p < 0.05$.

See also Figure S1.

IgG1 memory B cells had germline V_H186.2 sequences and ~30% of the Trp33 to Leu mutation, suggesting that the affinity for NP of the majority of IgG1 memory B cells was between 5×10^5 M^{-1} and 5×10^6 M^{-1} (Figure S2A; Furukawa et al., 1999). These endogenous $CD38^+NP^+IgG1^+$ memory B cells were transferred into CGG in alum-primed mice and their differentiation capacity was evaluated. As shown in Figure S2B, endogenous $CD38^+NP^+IgG1^+$ memory cells also had a high propensity to differentiate into plasma cells, like the $NP^+B1\text{-}8^{hi}$ IgG1 memory B cells, thus validating the use of the adoptive-transfer approach for subsequent studies.

Antigen Nonexperienced IgG1 B Cells Have a More Limited Differentiation Potential

The above observations strongly suggest that the high differentiation capacity of IgG1 memory B cells to become plasma cells is one of the key determinants to explain the rapid kinetics of secondary Ab responses. To explain the unique properties of IgG memory B cells, two non-mutually-exclusive mechanisms have traditionally been postulated. In the BCR-intrinsic model, the unique IgG cytoplasmic tail is thought to be the primary factor (Kaisho et al., 1997; Martin and Goodnow, 2002), whereas in the second model, BCR-extrinsic changes, such as alterations in transcription factors that take place during priming (Good and Tangye, 2007), are invoked.

One way to test the first model would be to examine the in vivo behavior of IgG1-expressing B cells that had never encountered cognate antigen; however, such cells do not exist in the normal immune system. As a way around this conundrum, we generated IgG1-embryonic stem cells (ESCs) by nuclear transfer from NP^+IgG1^+ memory B cells derived from C57BL/6 mice, with the idea that the prerearranged and pre-class-switched Ig heavy

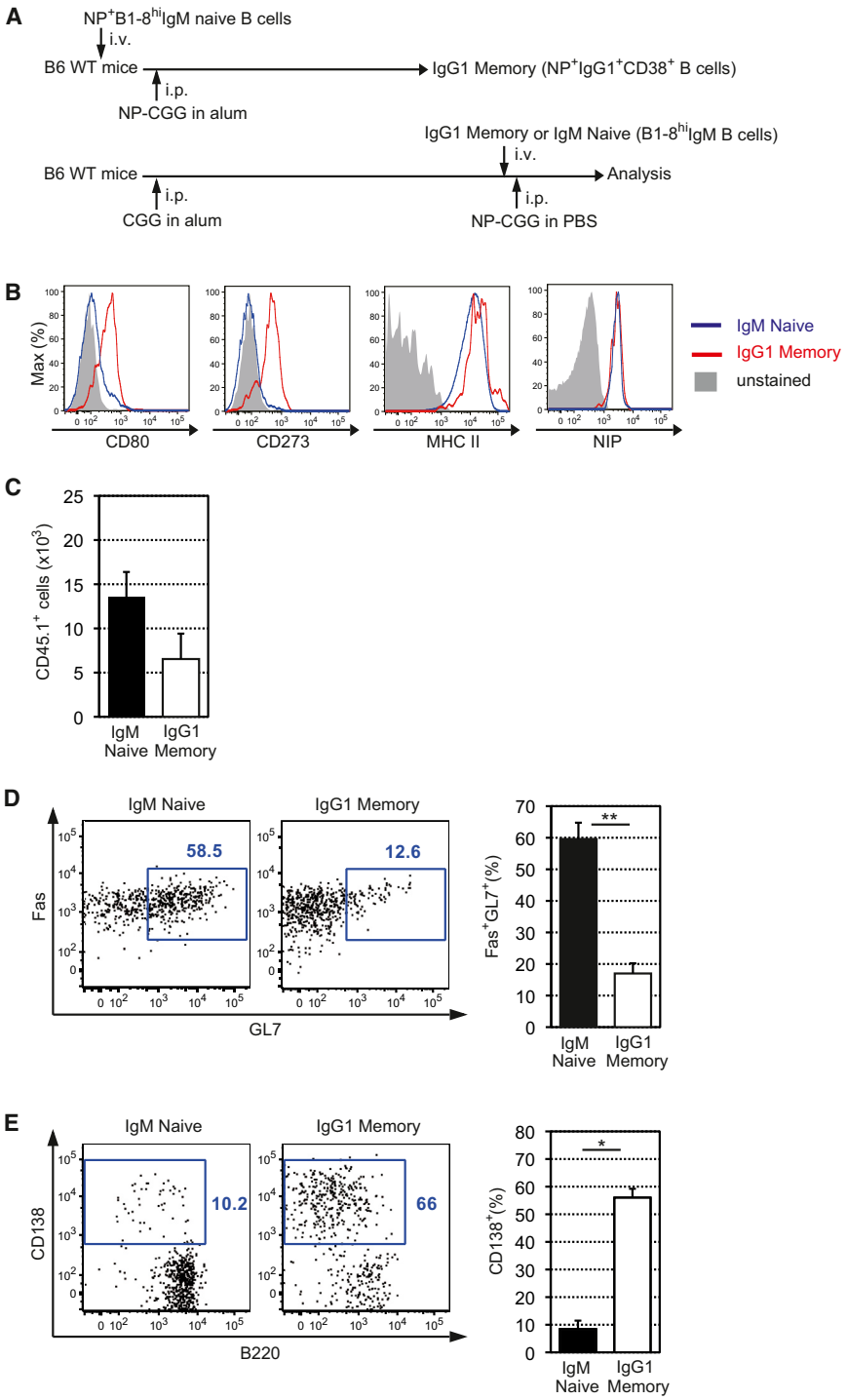

Figure 2. IgG1 Memory B Cells Have a High Propensity to Differentiate into Plasma Cells

(A) Schematic illustration of the transfer experiment.

(B) Expression of the indicated molecules was analyzed by flow cytometry. Blue lines indicate the expression by mature follicular NP^+CD38^+ IgM naive B cells collected from B1-8^{hi} gene-targeted mice. Red lines represent the expression in NP^+CD38^+ IgG1 memory B cells generated from B1-8^{hi} B cells.

(C–E) IgM naive or IgG1 memory B cells were transferred to CGG in alum-primed B6 recipients. On the next day, soluble NP-CGG was administrated i.p. Four days after the rechallenge, spleen cells were analyzed by flow cytometry.

(C) $CD45.1^+$ donor cell numbers in spleen were calculated.

(D) The percentage of germinal center B cells (Fas^+GL7^+) among donor cells was determined. Representative flow cytometric data is shown. Numbers indicate the percentage of cells within the gate.

(E) Plasma cells derived from donor cells were analyzed by flow cytometry and their percentage is plotted on the right.

Flow cytometric data are representative of at least three experiments. Bar graph represents the mean ± SD (n = 3–5). $^{**}p < 0.01$, $^{*}p < 0.05$. See also Figure S2.

and λ light chain genes in their natural chromosome context would allow us to characterize such antigen-nonexperienced IgG1 B cells. Several independent IgG1-ESC lines were established and used for making chimeric mice. Among these lines, we used clone NTB2 throughout the following experiments. Consistent with a previous study on IgG1 gene-targeted mice (Waisman et al., 2007), development of the B cells derived from the IgG1-ESCs was disfavored (Figure S3A), presumably because of the competition with blastocyst-derived B cells, which would arise from precursors in the bone marrow that undergo sequential Ig gene rearrangement and ultimately express IgM. Nevertheless, NP^+IgG1^+ B cells were generated in the bone marrow and present in the periphery. We examined maturation stages of these NP^+IgG1^+ B cells in the spleen; like peripheral B cells derived from the previous IgG1 gene-targeted mice (Waisman et al., 2007), proportions of the immature ($CD23^{lo}CD21^{lo}$) and the marginal zone ($CD21^{hi}CD23^{lo}$) stages were decreased or increased, respectively, compared with blastocyst-derived B cells (Figure S3B). Then, to experimentally determine the affinity of the NP^+IgG1^+ B cells for NP, we cloned antibodies from single cells (Figures S3C and S3D) and determined the affinity for NP by using thermodynamic characterization methods, revealing that Ka was 1×10^8 M^{-1} (called B1-8^{g1} IgG1 naive B cells hereafter) (Figures S3E and S3F).

Having established the above model, we first compared expression of CD80, CD273, and MHC-II on mature follicular NP^+B1-8^{g1} IgG1 B cells with those of NP^+B1-8^{hi} IgM naive B cells; they were found to be identical (Figure 3B). The capacity of the NP^+B1-8^{g1} IgG1 or NP^+B1-8^{hi} IgM naive mature B cells to mount a recall response was examined by adoptive transfer

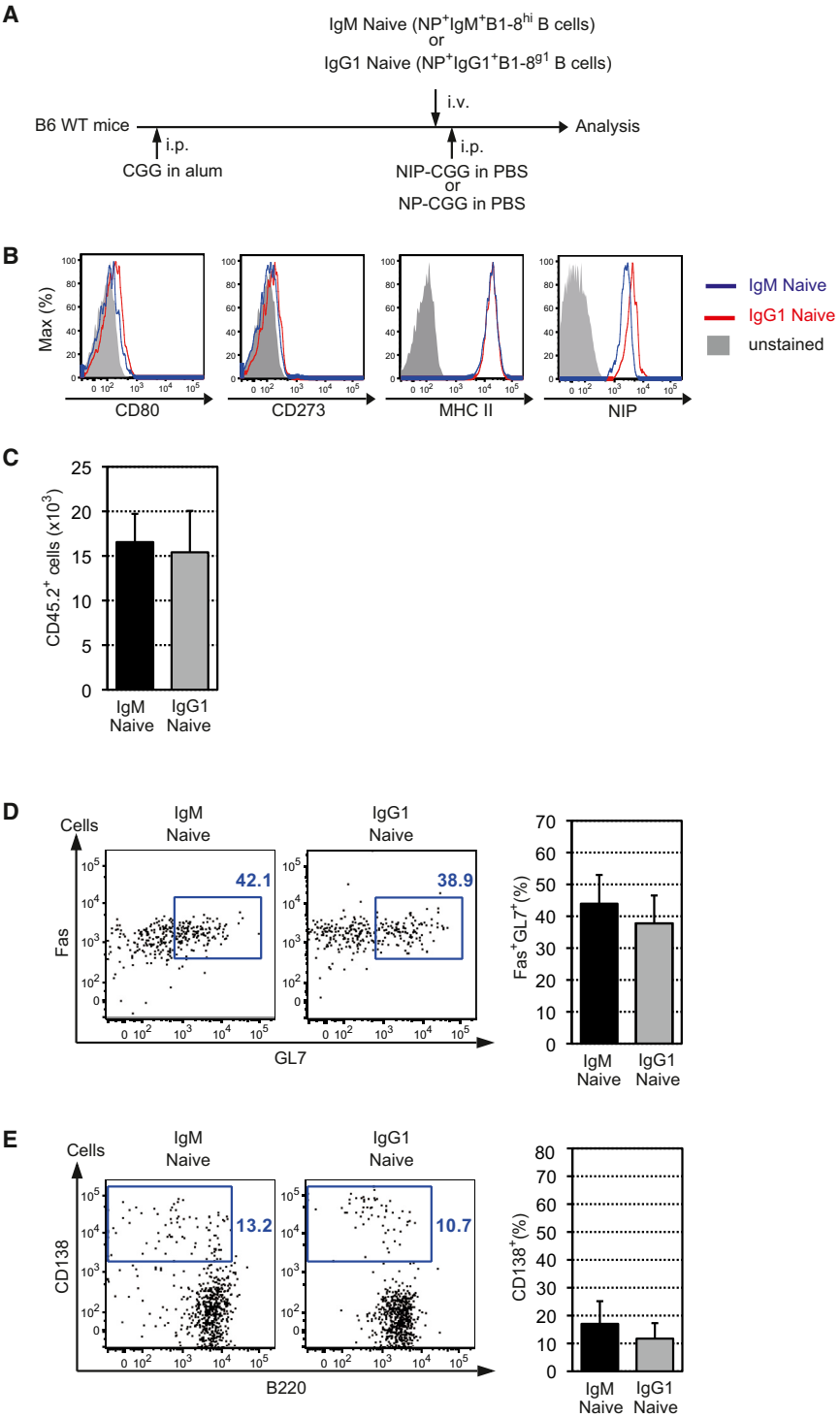

Figure 3. IgG1 Naive B Cells Differentiate in a Similar Fashion to IgM Naive B Cells

(A) Schematic illustration of the experimental protocol.

(B) Cell surface expression on mature follicular NP^+CD38^+ IgM naive B cells from B1-8^{hi} gene-targeted mice (blue line) or mature follicular NP^+CD38^+ IgG1 naive B cells from chimeric mice derived from IgG1-ES NTB2 (red line) was analyzed by flow cytometry. Gray histogram represents the unstained control.

(C–E) Mature follicular type cells of IgM naive B cells from B1-8^{hi} gene-targeted mice or IgG1 naive B cells from chimeric mice were sorted and transferred to CGG in alum-primed CD45.1 C57BL/6 mice. NIP-CGG or NP-CGG in PBS was injected i.p. on the next day. Four days later, donor cells were analyzed by flow cytometry. Analysis was carried out as described in the Figure 2 legend. Bar graph represents the mean ± SD (n = 3–5).

See also Figure S3.

of the same numbers of these mature follicular type B cells into CGG in alum-primed B6 mice, followed by immunization with NP-CGG or NIP-CGG in PBS, respectively (Figure 3A). Because the affinity for NP of B1-8^{g1} IgG1 B cells was ~20-fold higher than that of B1-8^{hi} IgM B cells, to minimize the effects of affinity difference on biological activity, we used NIP-CGG, instead of NP-CGG, for stimulation of B1-8^{hi} IgM B cells in these experimental settings; 4-hydroxy-5-indo-3-nitrophenyl acetyl (NIP) was reported to bind to the Ig composed of V_H186.2-DFL16.1-J_H2 and λ1, ~10-fold higher than NP (Imanishi and Mäkelä, 1973). As shown in Figures 3C–3E, NP^+B1-8^{g1} IgG1 B cells expanded to a similar extent, compared with NP^+B1-8^{hi} IgM B cells, and both of these B cell types underwent predominantly GC reactions rather than differentiation into plasma cells.

These observations suggest that expression of the membrane type IgG1 heavy chain, per se, is not sufficient to explain the heightened capacity of antigen-experienced IgG1 memory B cells to differentiate into plasma cells and that additional changes take place during in vivo priming, thereby contributing to such traits. This hypothesis predicts that NP^+B1-8^{g1} naive IgG1 B cells, after being antigen experienced, should also acquire the heightened differentiation capability at the memory stage. To test this prediction, NP^+B1-8^{g1} naive IgG1 B cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), transferred, and immunized (upper line in Figure 4A). Then, we purified the resultant NP^+CD38^+ IgG1 B cells, almost all of which had lost CFSE, fitting the definition of memory cells, having responded to antigenic stimulation and retained CD38 expression (Figure 4B; Tarlinton, 2006). We compared their in vivo differentiation capability with parental mature follicular NP^+B1-8^{g1} naive IgG1 B cells (lower line in Figure 4A). As shown in Figure 4C, the antigen-experienced memory IgG1 B cells manifested higher differentiation activity, compared with parental naive IgG1 B cells.

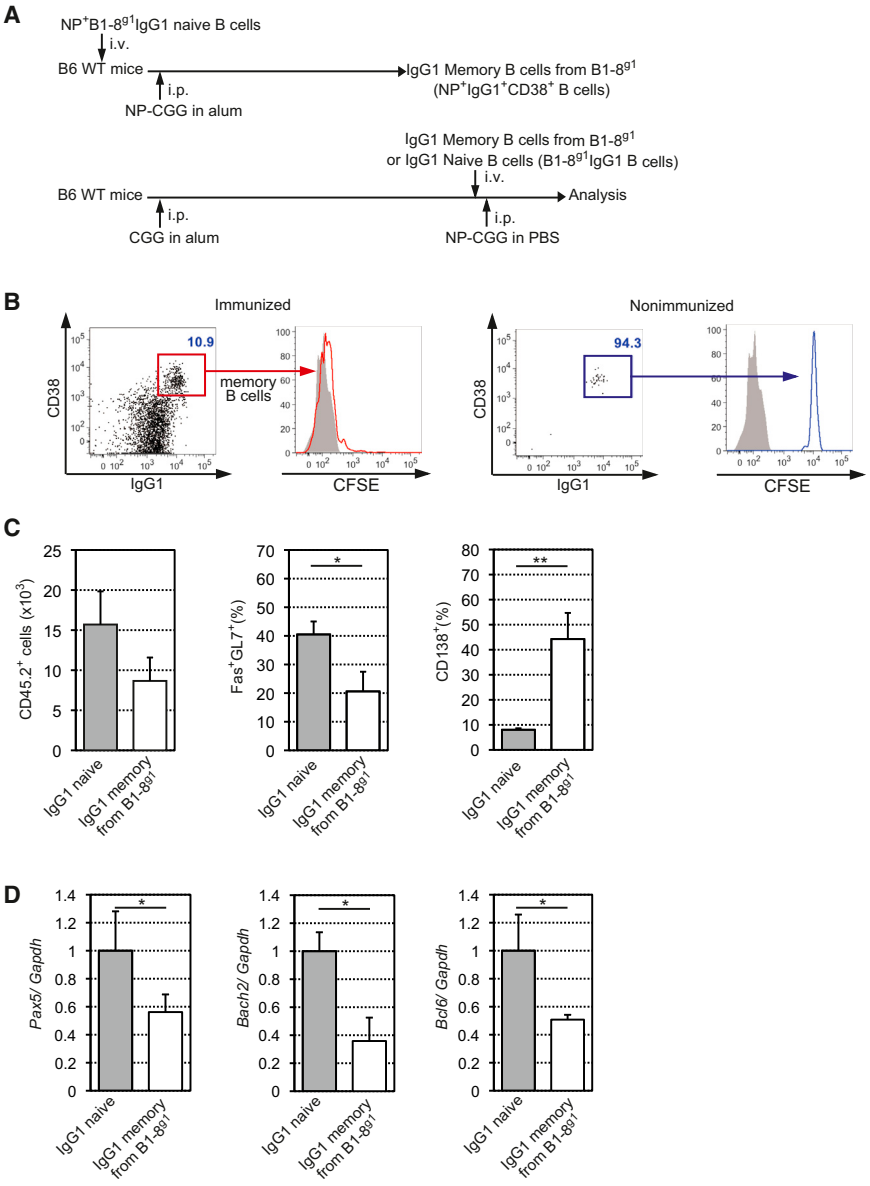

Figure 4. Antigen Experience Affects Differentiation Activity of IgG1 B Cells

(A) Schematic illustration for the generation of antigen-experienced NP$^+$IgG1$^+$ memory B cells and the transfer experiment.

(B) B cells collected from mature follicular naive IgG1 B1-8^{g1} B cells were labeled with CFSE and transferred to CD45.1 C57BL/6 mice. The mice were immunized with NP-CGG in alum i.p. or not. The donor cells were analyzed for the expression of CFSE, CD38, and IgG1 by flow cytometry 20 days later. Gray histogram represents CFSE-unlabeled recipient B cells. Data are representative of two independent experiments.

(C) An antigen-experienced NP$^+$IgG1$^+$ memory or parental NP$^+$IgG1$^+$ naive B cells were purified and transferred to CGG in alum-primed CD45.1 C57BL/6 mice, and differentiation ability was assessed by flow cytometry 4 days later after soluble NP-CGG rechallenge as described in the Figure 2 legend.

(D) The amount of each mRNA in NP$^+$IgG1$^+$ naive B cells and antigen-experienced NP$^+$IgG1$^+$ memory B cells was measured by qRT-PCR.

Data are representative of at least two independent experiments. Bar graph represents the mean ± SD (n = 3–5). **p < 0.01, *p < 0.05.

Pax5 and Bach2 Are Reduced in IgG1 Memory B Cells

The above results suggested the importance of BCR-extrinsic changes. To gain insights into what these might be, we first compared the amount of key transcription factors for plasma cell differentiation between NP$^+$B1-8^{hi} memory IgG1 and mature follicular NP$^+$B1-8^{hi} naive IgM B cells. Blimp-1, IRF-4, and XBP-1 are required for plasma cell differentiation (Iwakoshi et al., 2003; Klein et al., 2006; Martins and Calame, 2008), whereas factors such as Bcl-6, Pax5, and Bach2, which are found in GC or activated B cells, but not in plasma cells, are known to suppress these processes (Basso and Dalla-Favera, 2012; Cobaleda et al., 2007; Igarashi et al., 2007). The plasma cell differentiation factors (Blimp-1, IRF-4, XBP-1) were expressed at similar amounts in the two cell types (Figure 5A), whereas the amounts of Pax5, Bach2, and Bcl-6, were significantly reduced in NP$^+$B1-8^{hi} memory IgG1 B cells (Figure 5B). Given that *Bcl6* ablated mice can generate normal secondary anti-NP IgG1 responses (Toyama et al., 2002), involvement of Bcl6 in the enhanced plasma cell differentiation capability would seem unlikely, and therefore we focused on Pax5 and Bach2. Reflecting the amount of RNA, expression of Pax5 and Bach2 proteins was also decreased in NP$^+$B1-8^{hi} memory IgG1 B cells compared to NP$^+$B1-8^{hi} IgM naive B cells (Figure 5C). In contrast to Bach2, reduction of Pax5 protein was modest.

As shown in Figure 4D, the antigen-experienced memory IgG1 B cells derived from B1-8^{g1} B cells showed reduced expression of Pax5, Bach2, and Bcl6 compared with parental NP$^+$B1-8^{g1} naive IgG1 B cells.

Reduction of Bach2 in IgG1 Memory B Cells Promotes Plasma Cell Differentiation

To test whether reduction of Pax5 and Bach2 induces IgG1 memory B cells to preferentially differentiate into plasma cells, we examined the effects of reduction of these factors in another context, in IgM naive B cells, on their differentiation to plasma cells. Prestimulated B1-8^{hi} naive IgM B cells were transduced with gene-silencing retroviral constructs (Figure 6A) and transferred into C57BL/6 mice, which were then immunized with NP-CGG in alum. As demonstrated in Figure 6B, there was a higher proportion of plasma cells from *Bach2* silenced B cells compared with mock-transduced B cells, whereas there was no significant enhancement from *Pax5* silenced B cells. To further test the importance of Bach2 in IgG1 memory B cells,

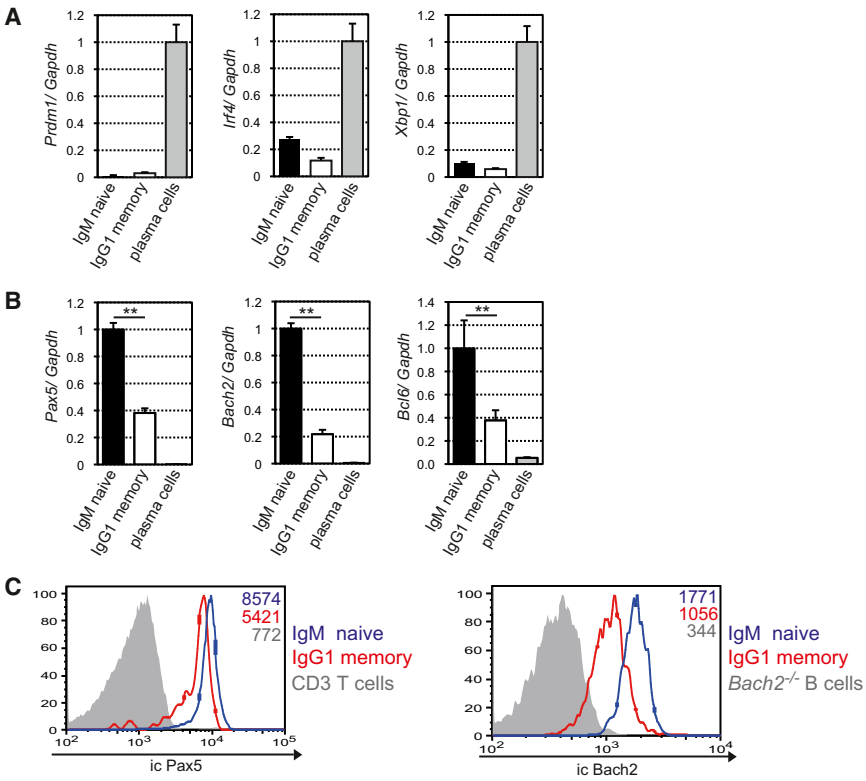

Figure 5. Expression of Bach2 Is Reduced in IgG1 Memory B Cells

(A and B) Mature follicular naive NP$^+$IgM$^+$ B cells were sorted from B1-8^{hi} mice. NP$^+$CD38$^+$B1-8^{hi} IgG1 memory B cells were isolated as shown in Figure 2A. The amount of each mRNA in these cells was measured by qRT-PCR. The data were normalized to the amount of *Gapdh*. As a control, CD138$^+$ plasma cells were sorted from splenocytes of NP-CGG in alum-immunized C57BL/6 mice. Bar graph represents the mean ± SD. The data are representative of at least three independent experiments.

(C) The amount of Pax5 or Bach2 protein was measured by intracellular flow cytometric analysis. Mature follicular naive NP$^+$IgM$^+$ B cells were prepared from B1-8^{hi} mice. NP$^+$CD38$^+$B1-8^{hi} IgG1 memory B cells were prepared as Figure 2A. Then, these B cells were fixed and permeabilized with paraformaldehyde followed by staining with Pax5 antibody or Bach2 antibody. CD3$^+$ T cells were used as a negative control for Pax5. Spleen B cells from Bach2-deficient mice (*Bach2*$^{f/f}$ × *Cd79a-cre*$^{KI/wt}$) were used as a negative control for Bach2. The numbers in each profile indicate geometric mean fluorescence intensity (gMFI) of each population. The data are representative of at least three independent experiments.

**p < 0.01. See also Figures S4 and S5.

we examined the effects of haploinsufficiency of Bach2 on plasma cell differentiation by using NP$^+$B1-8^{hi} memory IgG1 B cells from *Bach2*$^{flox/+}$ × *ERT2-cre* mice. As shown in Figures 6C and 6D, the haploinsufficient memory B cells manifested increased differentiation activity. Thus, our data, together with the previous evidence that Bach2 binds to the Blimp1 promoter region and represses its transcription (Ochiai et al., 2006), demonstrate that the reduction of Bach2 is probably a direct cause for conferring high plasma cell differentiation propensity on IgG1 memory B cells.

mTOR Is a Potential Regulator for Bach2 Repression

To address the potential mechanisms by which antigen-experienced B cells induce repression of Bach2, we focused here upon the early regulatory events initiated by BCR stimulation. To do this, we employed the in vitro experimental system. In vitro stimulated B cells (anti-BCR+IL-4+anti-CD40) underwent repression of Bach2. Among various inhibitors that are well known to modulate the transcription processes in B cells, rapamycin and API-2 (an AKT inhibitor) significantly inhibited the Bach2 repression (Figures 7A and S6A). Because S6 kinase, one of the readouts of mammalian target of rapamycin (mTOR) activation, was activated in in vitro stimulated B cells (Figure 7A), our results demonstrate that antigen-stimulated B cells activate a phsophatidyl inositol 3′-OH kinase (PI3K)-AKT-mTOR pathway, which in turn is important for initiation of Bach2 repression. In vivo data support this conclusion: both antigen-induced phosphorylation of S6 protein and Bach2 repression were inhibited by rapamycin treatment (Figure 7B).

Because the transcription factor Foxo1 is known to function in the AKT pathway (Rao et al., 2012), we also examined the involvement of Foxo1 by in vitro experiments. Stimulation-mediated Bach2 repression was further enhanced by treatment of a Foxo1 inhibitor (Figures 7C and S6B; Nagashima et al., 2010). Conversely, when B cells were retrovirally transduced with a constitutively active form of Foxo1 (Foxo1-CA) (Tang et al., 1999), this Bach2 repression was reduced (Figures 7D and S6C). These results suggest that Foxo1 could be a potential modulator for Bach2 repression in the PI3K-AKT-mTOR pathway.

DISCUSSION

In this study, we began to address a long-standing question regarding the mechanisms that govern heightened secondary IgG1 antibody responses. We were able to analyze antigen-nonexperienced IgG1 B cells by establishing IgG1-ESCs via nuclear transfer from NP$^+$IgG1$^+$ memory B cells. Our data show that the IgG1 cytoplasmic region, per se, is not sufficient to explain the unique properties of antigen-experienced IgG1 memory B cells and that changes in the amounts of transcription factors occur during priming, thereby contributing to the heightened differentiation activity of the resultant memory B cells. These conclusions are supported by the following three lines of evidence. First, in contrast to antigen-nonexperienced IgG1 B cells, IgG1 memory B cells had a high propensity to differentiate into plasma cells. Second, expression of the Pax5 and Bach2 transcription factors was downregulated in the IgG1 memory B cells. Third, by further reducing the expression of Bach2 in IgG1 memory B cells, their differentiation into plasma cells was enhanced.

Based on in vitro analysis of human memory B cells, it is often assumed that IgG-type memory B cells proliferate more rapidly

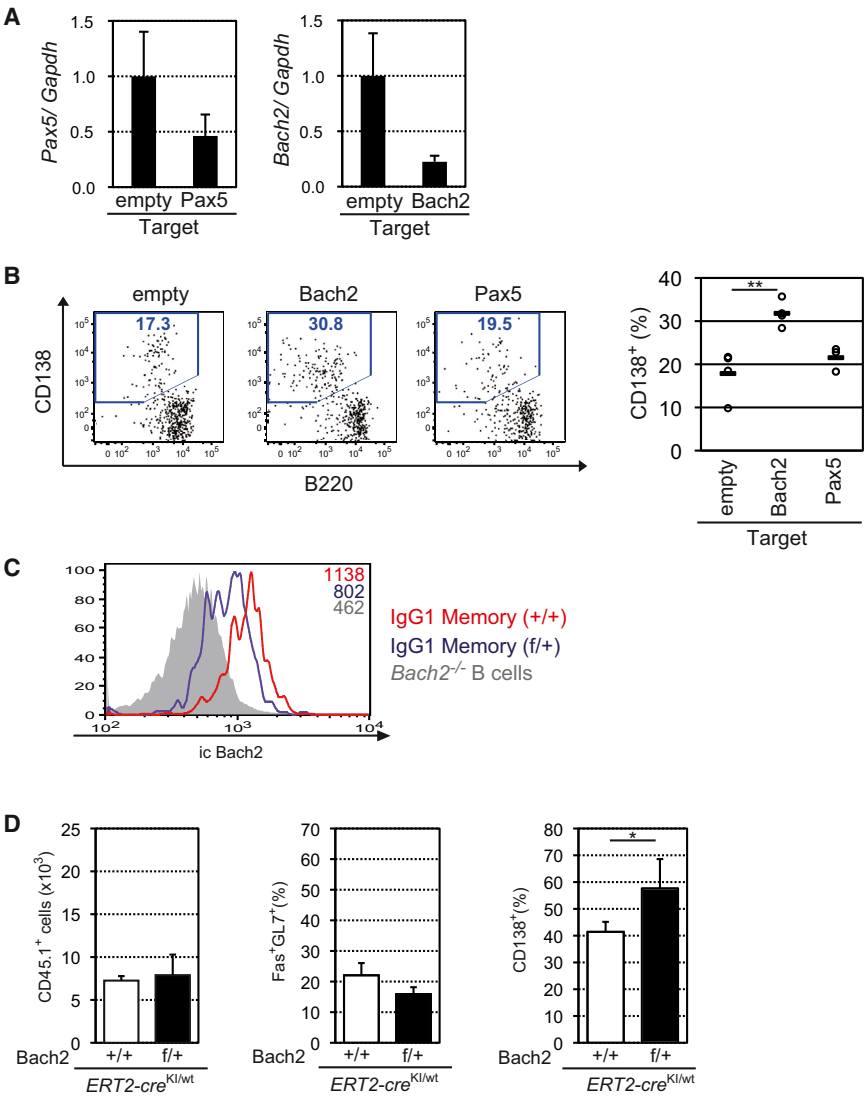

Figure 6. Reduced Expression of Bach2 Facilitates Plasma Cell Differentiation

(A) Prestimulated (see Experimental Procedures) B1-8^{hi} IgM B cells were transduced with retroviruses carrying gene silencing constructs for Bach2, Pax5, or empty vector, and further cultured for another 2 days. The transduced GFP^{+} cells were sorted and gene silencing efficiency was evaluated by qRT-PCR. Bar graph represents the mean ± SD (n = 3).

(B) B1-8^{hi} IgM B cells transduced with the indicated retroviruses were transferred to naive C57BL/6 mice followed by immunization with NP-CGG in alum. After 4 days, the donor cells in the spleen gated on GFP^{+} cells were analyzed for the expression of CD138 and B220 by flow cytometry. The bar in the graph indicates the mean ratio.

(C and D) Antigen-experienced NP^{+}CD38^{+}IgG1^{+} memory B cells from *Bach2*$^{+/+}$ × *ERT2-cre*$^{KI/wt}$ × B1-8^{hi} or *Bach2*$^{f/+}$ × *ERT2-cre*$^{KI/wt}$ × B1-8^{hi} mice were generated as illustrated in Figure 2A. Mice were treated with 2 mg tamoxifen by p.o. for 3 consecutive days. At 2 days after last administration, the cells were collected from spleen cells and used for the experiments.

(C) NP^{+}CD38^{+} IgG1 memory B cells from *Bach2*$^{+/+}$ × *ERT2-cre*$^{KI/wt}$ × B1-8^{hi} or *Bach2*$^{f/+}$ × *ERT2-cre*$^{KI/wt}$ × B1-8^{hi} mice were analyzed for measuring the expression of Bach2 by intracellular flow cytometric analysis as described in Figure 5C legend.

(D) The above IgG1 memory B cells were transferred to CGG in alum-primed C57BL/6 recipients. On the next day, soluble NP-CGG was administrated i.p. At 4 days after the rechallenge, spleen cells were analyzed by flow cytometry as described in Figure 2 legend.

The data represent three independent experiments. Bar graph represents the mean ± SD (n = 3–5). **p < 0.01, *p < 0.05.

than do naive IgM B cells, thereby at least partly accounting for the heightened secondary antibody response (Tangye et al., 2003). However, the in vivo expansion capacity of mouse IgG1 memory B cells shown here appears instead to be somewhat less than that of the naive IgM B cells. This disparity might simply reflect species differences between human and mouse. Alternatively, it could be caused by the fact that the human in vitro culture system with CD40L, IL-2, and IL-10 (Arpin et al., 1997) does not adequately recapitulate the in vivo GC processes, thus lacking the detection of high rate of cell division of IgM centroblasts that normally occurs in vivo. Because Pax5 and Bach2, key transcription factors for generating GC B cells, are similarly expressed at high amounts in both human and mouse IgM naive B cells (Robichaud et al., 2004; Sasaki et al., 2000), it is reasonable to anticipate that upon encounter with appropriate T cell-dependent antigens, human IgM naive B cells would enter the GC pool where they would undergo extensive proliferation in this in vivo context. Thus, this GC-dependent proliferation of human IgM naive B cells would be missed in the in vitro assay system. By contrast, assuming that human IgG1 memory B cells, like their mouse counterparts, prefer to differentiate into plasma cells rather than entering the GC pool, this GC-dependent effect would be minimized.

Another difference between our results and those from a previous study is the biological activity of the antigen-nonexperienced IgG1-type B cells, which we have demonstrated to have similar in vivo activity to IgM-type naive B cells. By contrast, the previous study comparing antigen-nonexperienced B cells expressing a transgenic anti-HEL chimeric IgM-IgG1 BCR or a transgenic IgM BCR shows that although the initial proliferation of IgM-IgG1 B cells is similar to that of the IgM B cells, the cytoplasmic IgG1 tail markedly enhances survival at the plasmablast stage (Martin and Goodnow, 2002). One explanation could be that the affinity difference for antigens in the two experimental systems (the affinity of the V_H186.2 germline-type BCR for NP is about Ka = 5 × 10^5 M^{-1}, whereas the transgenic BCR for hen egg lysozyme [HEL] is Ka = 2 × 10^{10} M^{-1}) might cause such differential outcomes (Padlan et al., 1989). It is well known that high-affinity antigen generates a robust extrafollicular plasmablast response, whereas low-affinity antigen

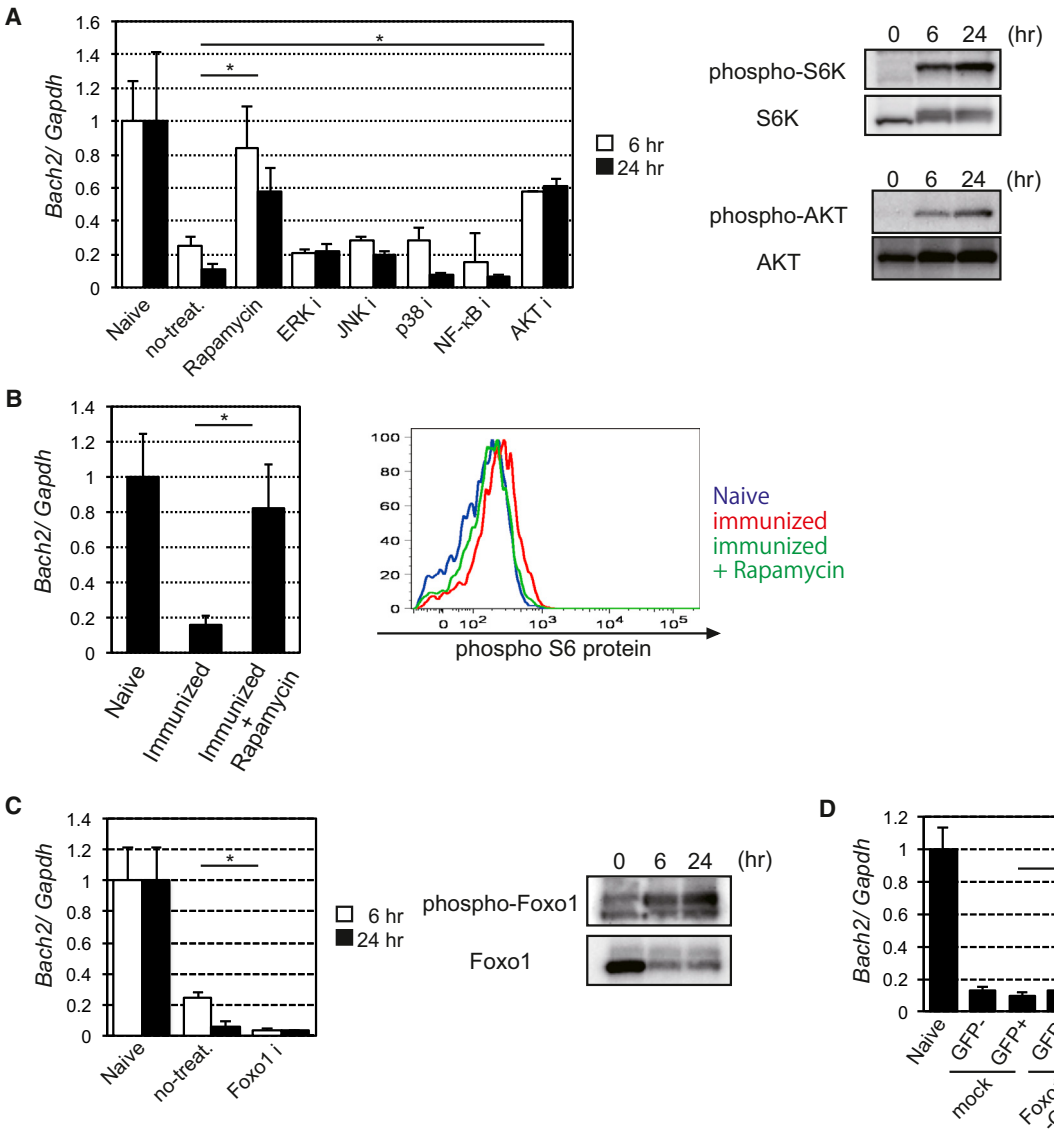

Figure 7. Bach2 Repression Was Suppressed by Rapamycin

(A and C) Purified C57BL/6 spleen B cells were treated with each inhibitor (shown on x axis) for 60 min: mTOR, Rapamycin (20 nM); ERK, PD98059 (50 μM); JNK, SP600125 (10 μM); p38, SB203580 (10 μM); NF-κB, IKK inhibitor (10 μM); AKT, API-2 (20 μM); or Foxo1, AS1842856 (1 μM). These cells were further cultured with CD40 antibody (2 μg/ml), IL-4 (10 ng/ml), and IgM antibody (1 μg/ml) for 6 or 24 hr in the presence of each inhibitor. The amount of Bach2 mRNA in these cells was measured by qRT-PCR. The data were normalized to the amount of *Gapdh*. The phosphorylation status of S6K, AKT (Ser473), and Foxo1 (Ser256) in the absence of inhibitors was analyzed by immunoblotting.

(B) Naive B cells from CD45.1 B1-8^{hi} gene-targeted mice were transferred to C57BL/6 mice followed by immunization with NP-CGG in alum. Rapamycin (300 μg/kg) or PBS (control) were injected i.p. on day −1 and day 0. At 24 hr after Ag injection, phosphorylation status of S6 protein of immunized donor B cells and parental naive B cells was measured by intracellular flow cytometric analysis. The donor B cells of immunized mice or naive B cells from B1-8^{hi} gene-targeted mice were collected by sorting and the amount of Bach2 mRNA was measured by qRT-PCR.

(D) Purified C57BL/6 spleen B cells were prestimulated with CD40 antibody (2 μg/ml), IL-4 (10 ng/ml), and IgM antibody (1 μg/ml) for 1 day and infected with a mock or Foxo1-CA retrovirus. The infected cells were further cultured for 48 hr and infected GFP$^+$ and noninfected GFP$^-$ cells were sorted. The amounts of Bach2 mRNA were measured by qRT-PCR. Bar graph represents the mean ± SD. The data were the representative of at least three independent experiments. *$p < 0.05$. See also Figure S6.

directs the B cells into the GC reaction (Paus et al., 2006). In this regard, our antigen-nonexperienced IgG1 B cells prefer to enter the GC pathway. Thus, it is possible that the cytoplasmic IgG1 tail might play a critical role at the extrafollicular plasmablast, but not the GC, stage. Another explanation is that the use of an adjuvant can affect the outcome. In the HEL system, complete Freund's adjuvant was used for immunization (Martin and Goodnow, 2002), whereas in our experiments a soluble antigen was injected into carrier protein-primed mice. Thus, for instance, in contrast to the HEL case, contributory effects

of dendritic cells (DCs) and inflammatory cells to T cells and B cells could be minimized in our assay system. If so, we consider this an advantage, because it provides a simpler system with fewer variables to compare the responses of naive versus memory B cells.

Pax5 controls the gene expression program of B cells, whereas Blimp1 orchestrates the transcription program of terminally differentiated plasma cells (Shapiro-Shelef and Calame, 2004). Despite such an inverse correlation, the reduction of Pax5 observed in IgG1 memory B cells appears not to directly influence the heightened differentiation capability of IgG1 memory B cells. Instead, our data suggest that reduction of Bach2 plays a more direct role. Three lines of previously reported evidence also support this idea. First, it is reported that the loss of Pax5 alone in mouse mature B cells is not sufficient for induction of terminal plasma cell differentiation (Horcher et al., 2001). Second, on the other hand, absence of Bach2 induces a striking increase in the onset and rate of plasma cell differentiation upon in vitro lipopolysaccharide (LPS) stimulation, and this Bach2 response is strongly dose dependent (Muto et al., 2010). Finally, Bach2, but not Pax5, binds directly to the Blimp1 gene, *PRDM1*, thereby repressing it (Ochiai et al., 2006; Shapiro-Shelef and Calame, 2004).

In regard to the Bach2 repression mechanism, our in vitro data suggest that initiation of this repression is induced by mTOR pathway. In vivo rapamycin treatment also supports this idea, although we cannot completely exclude the possibility that the observed in vivo effects might not be B cell intrinsic, but rather might be mediated by some other cells of the immune system. In the case of $CD8^+$ T cells, sustained PI3K-AKT-mTOR activity is known to inhibit Foxo1, which acts as a molecular switch to simultaneously induce T-bet expression, thereby promoting the terminal differentiation of effector T cells (Rao et al., 2012). Thus, it is reasonable to speculate that B cells might utilize the similar mechanism; antigen-experienced B cells repress Bach2 expression by the sustained PI3K-AKT-mTOR activity, which brings these B cells into the predisposed state for Blimp-1 induction and subsequent plasma cell differentiation.

Recent reports demonstrate that, in contrast to IgG memory B cells, IgM memory B cells rather prefer to induce secondary GC reactions (Dogan et al., 2009; Pape et al., 2011), therefore prompting us to examine the status of Bach2 in IgM memory B cells. Correlating with such functional data, reduction of Bach2 mRNA and protein in NP^+ IgM memory B cells was limited compared with that observed in NP^+ IgG1 memory B cells (Figure S5). In regard to mechanisms causing differences in the levels of Bach2 between IgG1 and IgM memory B cells, the following three possibilities can be envisaged. First, given that IgM memory B cells are generated earlier than IgG1 memory B cells (Taylor et al., 2012), spending relatively a shorter time for the expansion phase (in the case of IgM memory cells) might limit the downregulation of Bach2. Second, the IgG1 tail might facilitate Bach2 downregulation during generation of IgG1 memory B cells. Finally, rather than induction for Bach2 repression, its maintenance mechanism could differ between IgM and IgG1 memory B cells; the established repressed state for Bach2 might be well maintained in IgG1 but not IgM memory B cells. Additional studies are underway to define which of the preceding possibilities is most likely.

Although our experiments do not rule out a role for the IgG1 cytoplasmic domain in the unique traits of IgG1 memory B cells, they demonstrate that this domain alone is insufficient to confer the heightened differentiation activity of IgG1 memory B cells. In addition, our data strongly support the concept that antigen experience induces repression of Bach2 (one of the BCR-extrinsic changes) in IgG1 memory B cells, which in turn contributes to rapid humoral recall responses.

EXPERIMENTAL PROCEDURES

Mice

$B1\text{-}8^{hi}$ gene-targeted mice, $C\gamma1$-*cre* gene-targeted mice, iDTR mice, and *Cd79a-cre* mice were provided by M. Nussenzweig (Shih et al., 2002), K. Rajewsky and S. Casola (Casola et al., 2006), A. Waisman (Buch et al., 2005), and M. Reth and E. Hobeika (Hobeika et al., 2006), respectively. Rosa26-*ERT2-cre* gene-targeted mice were purchased from Taconic Firm. B1-8 germline gene-targeted ($B1\text{-}8^{ge}$) mice and flox-Bach2 mice were generated with Bruce4 ESCs. To generate IgG1-ESC mice, the nuclei of $NP^+Ig\lambda^+CD38^+$ IgG1 memory B cells derived from C57BL/6 mice were transferred to nucleus-expelled BDF1 unfertilized eggs. Chimeric mice were generated by the ESC injection into blastocysts of BALB/c mice. All the mice were maintained under specific-pathogen-free conditions. The protocols for animal experiments were approved by the RIKEN Animal Research Committee.

Immunization

For primary responses, mice were injected intraperitoneally (i.p.) with 100 μg of NP-CGG precipitated with Imject alum (Thermo Fisher Scientific). For recall responses, 50 μg of soluble NP-CGG in PBS was injected i.p.

In Vivo Cell Depletion

Mice were injected i.p. with 100 ng of diphtheria toxin (4 ng DT/g body weight; Sigma) for 3 consecutive days.

Adoptive Transfers

For generating memory B cells, purified $CD45.1^+B1\text{-}8^{hi}$ B cells containing 1×10^5 NP-binding B cells were transferred intravenously (i.v.) into C57BL/6 mice. On the next day, 100 μg of NP-CGG precipitated with Imject alum was injected i.p. Thirty days later, spleen cells were collected and NP-specific IgG1 memory B cells were sorted after the depletion of dump gate-positive cells via magnetic beads column system. NP-binding naive B cells were sorted from B1-8 gene-targeted mice. Sorted 5,000 NP-binding cells were transferred to recipient mice i.v. and the mice were boosted with 50 μg of soluble NP-CGG in PBS i.p. on the next day.

Flow Cytometric Analysis

Single-cell suspensions lysed of red blood cells were stained with fluorochrome-conjugated antibodies. For intracellular staining, the cells were fixed and permeabilized with a Foxp3 staining kit (eBioscience) followed by staining with anti-Pax5 (Santa Cruz Biotechnology), Bach2 antibody (established in our laboratory), or phosho-S6 ribosomal protein (Cell Signaling Technology). The stained cells were analyzed by FACSCantoII (BD Bioscience).

In Vitro Culture

Purified naive B cells from spleens of C57BL/6 mice were pretreated with various inhibitors for 60 min. The cells were further cultured with CD40 antibody (2 μg/ml; BioLegend), IL-4 (10 ng/ml; R&D), and IgM antibody (1 μg/ml; Jackson ImmunoResearch) at 37°C under 5% CO_2 for 6 or 24 hr in the absence or presence of each inhibitor. For retrovirally gene transfer experiments, prestimulated B cells were infected with retrovirus and further cultured for 48 hr. The GFP^+ or GFP^- cells were sorted for the assay via FACSAria.

Enzyme-Linked Immunosorbent Assay

NP_{20}-BSA or NP_1-BSA (for high affinity) was used as the capture antigen. After incubation with serially diluted sera, detections were done with horseradish peroxidase-conjugated anti-mouse IgM or IgG1 antibodies (SouthernBiotech)

and SureBlue (KPL) substrate. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad).

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) and DNaseI (Invitrogen)-treated RNA was reverse transcribed with Super Script III (Invitrogen). Quantitative PCR was performed with SYBR Green (Invitrogen) and the ABI StepOnePlus realtime PCR system (Applied Biosystems).

RNAi Gene Silencing Assay

For generating the gene silencing vector, the target sequence was inserted to the pMYs retroviral vector via the BLOCK-iT polII miR RNAi system (Invitrogen). Prestimulated B cells purified from B1-8^{hi} gene-targeted mice were infected and GFP^{+} cells were analyzed by flow cytometry.

Measurement of BCR Affinity

To examine NP-binding abilities of BCRs, their Fab regions were bacterially expressed. The affinity was measured by isothermal titration calorimetry (ITC) method with Fab proteins and NP-Cap (Biosearch Technologies).

Statistical Analysis

Statistical analyses were performed by a two-tailed unpaired Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.06.011.

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